EARLY SEEDLING GROWTH TEST WITH A TERRESTRIAL PLANT SPECIES

1. TEST OBJECTIVE

To assess the toxicity of a soil or test material to a terrestrial plant species and determine the effects on seedling survival, seedling height, root length, dry weight, or a combination of these endpoints.

2. TEST ARTICLE

2.1 Description/Identification

The test article is a soil sample. Unless otherwise specified, the test article is supplied by the client. Adequate chemical and/or biological specifications with special reference to hazardous properties and storage conditions are also supplied by the client. When available, information on the stability, composition, or other characteristics which define the test article are on file with the client.

2.2 Sample Preparation

The soil sample should be screened through an appropriately sized sieve (e.g., 2.0 mm) to remove large particles and indigenous organisms, and then homogenized before being placed in the test vessels.

3. EXPERIMENTAL DESIGN

3.1 Test Organisms

3.1.1 Species

Seeds from an appropriate terrestrial plant species, such as lettuce (*Lactuca sativa*), are used.

3.1.2 Source

Seeds are obtained from a commercial seed company, as specified in the report. Information on seed lot, year/growing season in which seeds were collected, and germination percentage should be provided by the seed source. Only untreated (not treated with fungicide, repellants, etc.) seeds are acceptable for use in toxicity testing.

3.1.3 Storage Conditions

Seeds are stored in the dark at 4°C in air-tight, waterproof containers.

3.1.4 Organism Condition at Test Initiation

Seeds are inspected for hull integrity, and empty hulls or seeds with split, cracked or damaged hulls are disposed. Seeds should be graded by size either visually or with wire mesh sizing screens. A toxicity test is initiated with seeds that are approximately equal in size.

3.2 Test Concentration Series

The test species is exposed in replicate chambers to soil samples and to a laboratory or reference sample control. Screening assays may be conducted on whole soil samples. Alternatively, a definitive (multi-concentration test) may be conducted on a sample using a laboratory or reference soil to prepare the test concentrations.

3.3 Test Vessels and Test Volume

Test vessels are appropriately sized pots which do not restrict seedling growth. Selection of the type and size of test vessels is dependent on the species to be tested. For testing with *L. sativa*, test vessels are typically 6-inch diameter polypropylene pots, approximately 1.5 L in volume.

The test volume will depend on the size of the test vessel. For *L. sativa* testing, approximately 1,500 g of air-dried control or test soil is typically used.

3.4 Test Organism Number and Test Initiation

The toxicity test is typically conducted with five replicates per control and test treatment, with 10 seeds sown per replicate. The seeds are arranged equally spaced in the pot and covered with a thin layer of soil. The surface of the soil is carefully moistened with deionized water, taking care not to disturb the planted seeds.

3.6 Test Environment

Test vessels are maintained in an environmentally controlled chamber at 25±1°C under wide spectrum fluorescent light, with a 16-hour light/8-hour dark photoperiod. Humidity is maintained at greater than 30 percent.

3.7 Test Observations

Germination is monitored daily. After approximately two weeks, the number of seedlings in each pot may be thinned to allow for unrestricted growth, while still maintaining enough biomass for dry weight measurements at test termination. The number of live plants per test vessel is monitored daily in addition to the temperature of the environmental chamber, the light level, and humidity.

Test vessels are watered daily, or as needed, with deionized water. It may be necessary to apply a liquid fertilizer, such as Hoagland nutrient solution, to stimulate adequate plant growth. If nutrient solution is applied, all test and control vessels receive equal amounts of solution. The addition of nutrient solution is documented on the test data sheets.

The test terminates after four weeks of exposure; however, the test length may be extended at the request of the client. At test termination, the number of surviving plants per replicate is recorded. The plants from each replicate are carefully extracted from the test vessel for seedling height, root length, and/or weight measurements. For dry weight analyses, all adhering soil is gently rinsed from the plant leaves and/or roots (depending on the project objectives) with deionized water, and the plants from each replicate are placed into pre-weighed, oven-dried pans, The plants are dried in an oven at 100°C for a minimum of 24 hours. The mean dry weight of the plants in each replicate is then determined by subtracting the weight of the pan from the combined dry weight of the pan with plants.

3.9 Test Acceptability

For a test to be acceptable, survival in the controls should be at least 90 percent.

3.10 Data Analysis

Statistical analyses can be performed on percent survival, seedling height, root length, and/or dry weight data to determine if a test treatment is significantly different from the control. For screening assays, a t-test or Wilcoxon's Rank Sum test is used depending on normality and equality of variance. Shapiro-Wilks test is used to test for normality, and the F-test for equality of variance is used to test the homogeneity of variance assumption. For definitive assays, an analysis of variance (ANOVA) and either Bonferroni's T-Test or Dunnett's Mean Comparison Test are used to analyze significance of effects. Depending on the distributional characteristics of the data generated, it may be necessary to use Steel's Many-One Rank Test or the Wilcoxon Rank Sum Test instead (US EPA 1994). An LC50 and/or EC50 may be calculated from a definitive test using the probit, moving average, and binomial methods as described by Stephan (1977). Depending on the nature of the data, other methods may be used including the Trimmed Spearman-Karber method, the probit approximation method of Litchfield and Wilcoxon (1949), SAS probit analysis (SAS Institute 1985), or graphical interpolation using the log concentration

vs. percent mortality and/or percent affected as described by APHA et al. (1995). The statistical methods are specified in the final report.

4. FINAL REPORT

The final report is prepared to contain, at a minimum, the following information:

- Objectives and procedures stated in the approved protocol, including any changes made to the original protocol
- Identity of the test article(s)
- Source of the control soil and/or reference soil and its chemical characteristics (if available)
- Any unforeseen circumstances that may have affected the quality or integrity of the study
- Signature of the project manager, senior technical reviewer, and quality control officer authorizing release of the report
- Location of all archived data and the original copy of the final report at EA

Items of data to be included in the report consist of experimental design and test performance, effects on general appearance of test organisms (if applicable), presentation of environmental parameters, survival data, and results of seedling height, root length, and/or dry weight determinations.

5. QUALITY ASSURANCE

5.1 Amendments to Protocol

Amendments to the authorized protocol established by EA or by the client are made only after proper authorization. Such authorization is achieved by completion of the Protocol Amendment Form by EA after consultation with the client.

5.2 Standard Operating Procedures

Unless otherwise specified, all procedures given in the protocol are subject to detailed Standard Operating Procedures (SOPs) which are contained in the SOP manuals of the participating

departments. These SOPs and protocols generally follow the type of requirements in the U.S. EPA's Good Laboratory Practice Standards (GLPs) (US EPA 1989).

5.3 Reference Toxicant

A reference toxicant test with an appropriate toxicant may be used as an internal quality check of the sensitivity of the test organisms. Testing is conducted at least once on each lot of seeds preferably conducted concurrently with the toxicity test. The results of each test are compared with historical species-specific toxicological information from reference toxicant tests performed at EA to determine if the results are within acceptable limits using the control charts outlined in US EPA (1993).

5.4 Quality Assurance Evaluation

The study described in this protocol may be subject to internal audit by EA's Quality Assurance Unit. A quality control officer is responsible for monitoring each study to assure the client that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with EA's QC program and EPA's GLPs.

5.5 Inspection by Regulatory Authorities

In the event of an inspection of EA by an outside authority during the course of the study, the client will be consulted before inspectors are permitted access to any of the project records or the experimental areas.

5.6 Archives

Copies of project-specific records shall be transferred to the client promptly after the project is completed or as negotiated and budgeted with each client. Original primary data are retained at EA for 5 years. Primary data include chain-of-custody records, laboratory data sheets, records, memoranda, notes, photographs, microfilm, and computer printouts that are a result of the original observations and activities of the study and which are necessary for the reconstruction and evaluation of the study report.

5.7 Location

Studies are conducted at the Ecotoxicology Laboratory of EA Engineering, Science, and Technology, Inc. at the Loveton Office in Sparks, Maryland.

6. SPECIFICATIONS OF THE EARLY SEEDLING GROWTH TEST

6.1 Basic References

American Public Health Association (APHA) American Water Works Association, Water Environment Federation. 1995. Standard Methods for Examination of Water and Wastewater, 19th or most recent version. APHA, Washington, D.C.

American Society for Testing and Materials (ASTM). 1994. Standard Practice for Conducting Early Seedling Growth Tests. ASTM Designation: E 1598-94, Philadelphia, Pennsylvania.

EA. 1996. Quality Control and Standard Operating Procedures Manual for EA□s Ecotoxicology Laboratory. Fifth Revision. EA Manual ATS-102. Internal document prepared by EA□s Ecotoxicology Laboratory, EA Engineering, Science, and Technology, Inc., Sparks, Maryland.

Litchfield, J.T., and F. Wilcoxon. 1949. A simplified method of Evaluating Dose/Effect Experiments. J. Pharmacol. Exp. Ther. 96:99-113.

US EPA. 1979. Methods for Chemical Analysis of Water and Wastes. EPA/600/4-79/020. U.S. Environmental Protection Agency, Washington, D.C.

US EPA. 1988. Protocols for Short Term Toxicity Screening of Hazardous Waste Sites. EPA/600/3-88/029. U.S. Environmental Protection Agency, Environmental Research Laboratory, Corvallis, Oregon.

US EPA. 1989. Toxic Substances Control Act (TSCA); Good Laboratory Practice Standards. Title 40 CFR Part 792. Fed. Regist. 54(158): 34034-34074.

US EPA. 1991. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Fourth Edition. EPA/600/4-90/027. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio.

US EPA. 1993. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Fourth Edition. EPA/600/4-90/027F. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio.

6.2 Test Specifications

Test organism:	Seeds of terrestrial plant species (e.g., lettuce-*Lactuca sativa*)
Temperature:	25±1°C
Light quality:	Wide spectrum fluorescent light
Photoperiod:	16-hours light/8-hours dark
Test container:	6-inch polypropylene pot, may vary depending on study design
Test volume:	1,500 g of air-dried control or test oil, may vary depending on study design
No. replicates:	5
No. organisms per vessel:	10 seeds sown
Test duration:	4 weeks, may be extended at the request of the client
Endpoints:	Survival, seedling height, root length, and/or dry weight
Test acceptability:	90 percent or greater survival in the control